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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

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| Applicant: | Foung *et al.* | Examiner: | Wortman, D. |
| Serial No.: | 09/728,720 | Art Unit: | 1648 |
| Filed: | December 1, 2000 | | |
| For: | PREVENTION AND TREATMENT OF HCV INFECTION EMPLOYING ANTIBODIES DIRECTED AGAINST CONFORMATIONAL EPITOPES | | |

Mail Stop: Amendment
Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Sir:

**DECLARATION UNDER 37 C.F.R. 1.132**

I, Steven K. H. Foung, declare as follows:

1. I am currently a Professor of Pathology at Stanford University School of Medicine.

2. I am a joint inventor of the subject matter disclosed and claimed in United States patent application Serial No. 09/728,720 filed December 1, 2000 and entitled "Prevention and Treatment of HCV Infection Employing Antibodies Directed Against Conformational Epitopes".

3. I have reviewed and understood United States patent application Serial No. 09/728,720, filed December 1, 2000 and entitled "Prevention and Treatment of HCV Infection Employing Antibodies Directed Against Conformational Epitopes," and have also reviewed and understood U.S. Pat. No. 5,308,750 (herein referred to as "Mehta et al.").

4. This Declaration is presented for the purpose of overcoming a rejection that the pending claims are obvious in light of the Mehta reference. The present Declaration is therefore intended to present scientific data that shows that the anti-HCV antibodies disclosed and claimed in the present claims are different from those that would be obtained by using an epitope defined by position 607-627 as an immunogen.

5. Experiments have been performed, under my direction, to determine which amino acid regions within HCV E2 contain the epitopes to which CBH-2, CBH-4G, CBH-5, CBH-7, CBH-8C, CBH-11, CBH-4B, and CBH-4D bind. A nucleic acid construct encoding amino acids 384-661 of HCV E2 protein from genotype 1b was generated by inserting the coding sequence for these amino acids into the pDisplay expression vector (Invitrogen). DNA encoding this portion of the E2 protein was prepared from HCV genotype 1b-positive serum by reverse transcription-PCR using *Pfu Taq* polymerase (Stratagene, La Jolla, Calif.) with HCV-specific oligonucleotide primers (forward$_{1b}$, 5'-AGATCTACCACCTACACGACGGGGGGGGC-3'; forward$_{1b411}$, 5'-AGATCTATCCAGCTCATAAACACCAACGGC-3'; reverse$_{1b}$, 5'-CTGCAGCTCTGATCTGTCCCTATCCTCCAAG-3'). Flanking BglII or PstI restriction sites in the primer sequences are underlined. Amplified DNA fragments were ligated into the pDisplay vector (Invitrogen) in frame with HA and c-*myc* as tags at the N and C termini, respectively. To map the epitope, a series of carboxyl- and amino-terminal deletion mutants of the E2 gene were synthesized by PCR and cloned into pDisplay vector with HA and c-myc tags (as described above). These E2 deletion constructs were then transfected into HEK293 cells and the protein extracts were analyzed by ELISA and confirmed by immunofluorescence assay. Expression of the proteins was verified using the HA monoclonal antibody. Amino acids 384-661 constitutes full length E2 without the transmembrane domain, and this portion of E2 is referred to herein as "full length E2". The fragments of amino acids 384-661 encoded by the E2 deletion constructs are referred to herein as "E2 deletion polypeptides".

6. To test whether tag proteins have an effect on antibody binding, intracellularly expressed 1a and 1b E2 proteins (amino acids 384-661) without tags were tested and shown to be without detectable differences from the tagged proteins by immunofluorescence assay. E2 amino acids 384-661 DNA sequencing of each E2 deletion construct confirmed the junction of deletion, and the expected sequences resulted in no frameshifts or premature terminations. The expression of the E2 deletion constructs was verified by Western blot analysis of cytoplasmic extracts of transiently transfected HEK293 cells by using a MAb to the tag-HA epitope.

7. For analysis of antibody binding to the HCV E2 deletion polypeptides, the E2 deletion constructs and a construct encoding full length E2 were individually transfected into HEK293T cells, which were then analyzed using flow cytometry. HEK293T cells were seeded to obtain 60 to 70% confluence by the following day. For transfection of a T-75 flask, a mixture of 40 μg of the plasmid DNA and 240 μg of PerFect Lipid Pfx-2 (Invitrogen) were combined in 1 ml of serum-free Dulbecco's MEM. After 4 h of incubation at 37°C, the transfection solution was replaced with 20 ml of complete medium. Flow cytometry assays were performed between 24 to 48 hours post-transfection. EGFP (Clontech, Palo Alto, Calif.) was used as an internal control for transfection efficiency. Transiently transfected cells were removed from the wells or flasks with Versene 1:5000 (Gibco BRL), washed with staining solution (PBS with 1% FCS and 0.1% sodium azide) and suspended at about $10^6$ cells/ml. Each test antibody at 10 ug/ml, in a total volume of 100 μl of staining solution (PBS plus 1% FCS and 0.1% sodium azide), was combined with $10^6$ viable cells expressing either full length E2, an E2 deletion construct, or containing a vector with no insert, resuspended in 100 μl of staining solution, and incubated at 4°C. for 45 minutes. After adding an additional 3 ml of staining solution, the cells were pelleted by centrifugation for 10 minutes at 500 x g at room temperature. The pellet was reserved and resuspended in 100 μl of FITC conjugated secondary antibody diluted as recommended by the manufacturer (Jackson Immunoresearch laboratories, West Grove, Pa.) in staining solution. After 45 minutes at 4°C., 900 μl of staining solution was added, and the cells were pelleted as above. The cells were then resuspended in 1 ml of fixative solution (3.8% formaldehyde in PBS), and the amount of HMAb bound to the surface of cells was analyzed on a FACScalibur flow cytometer (Becton-Dickinson, San Jose, Calif.). For two color staining, secondary antibodies were conjugated to R-phycoerythrin and fluorescence monitored at 606 nm while EGFP fluorescence was monitored at 545 nm.

8. The amino acids contained in each of the E2 deletion polypeptides are depicted schematically in Figure 1, together with names identifying the exact termini of the deletions. sf1b represents full length E2 (i.e., E2 amino acids 384-661, as used herein). The names of the E2 deletion constructs are interpreted as follows: A construct whose name consists of "pDN" followed by a number indicates a construct that encodes an E2 deletion polypeptide having an N-

terminal deletion extending from amino acid 384 to the amino acid immediately N-terminal to the amino acid at the position indicated by the number. For example, pDN411 encodes an E2 deletion polypeptide that extends from position 411-661; pDN510 encodes an E2 deletion polypeptide that extends from position 510-661. The constructs delete progressively larger portions of the N terminal region of E2. Generally the size of the deletion increases by an increment of ten amino acids.

12. The results of the flow cytometry analyses for antibodies CBH-2, CBH-4G, CBH-5, CBH-7, CBH-8C, CBH-11, CBH-4B, and CBH-4D are presented in the following tables, whose contents are described below.

| Antibody | Construct | Fluorescence (arbitrary units) |
|---|---|---|
| No AB | Vector | 13.67 |
| | Vector | 14.70 |
| PE only | Vector | 14.81 |
| | Vector | 15.23 |
| | | |
| CBH-7 | sf1b | 1267.66 |
| | pDN 480 | 853.73 |
| | pDN 490 | 931.51 |
| | pDN 500 | 24.43 |
| | pDN 520 | 25.75 |
| | | |
| CBH-2 | sf1b | 1192.03 |
| | pDN415 | 1098.54 |
| | pDN420 | 1074.01 |
| | pDN430 | 17.88 |
| | pDN440 | 17.02 |
| | | |
| CBH-5 | sf1b | 785.23 |
| | pDN415 | 1139.08 |
| | pDN420 | 957.08 |
| | pDN430 | 15.45 |
| | pDN440 | 14.99 |
| | | |
| CBH-8C | sf1b | 1144.11 |
| | pDN415 | 952.09 |
| | pDN420 | 992.43 |
| | pDN430 | 22.31 |
| | pDN440 | 20.23 |
| | | |
| CBH-11 | sf1b | 430.67 |
| | pDN415 | 286.64 |
| | pDN420 | 303.79 |
| | pDN430 | 15.04 |
| | pDN440 | 15.46 |

| Antibody | Construct | M1 means |
|---|---|---|
| CBH-4G | sf1b | 302.19 |
| | pDN490 | 360.14 |
| | pDN500 | 43.98 |
| | pDN510 | 24.07 |
| | pDN520 | 26.78 |
| | pDN530 | 25.14 |
| | pDN540 | 23.78 |
| | pDN550 | 22.78 |
| | pDN560 | 28.87 |
| | pDN570 | 35.32 |
| | pDN580 | 20.84 |

| Antibody | Construction | M1 means |
|---|---|---|
| CBH-4B | sf1b | 736.71 |
| | pDN570 | 169.61 |
| | pDN580 | 51.33 |
| | pDN590 | 113.37 |
| | pDN600 | 64.58 |
| | pDN610 | 14.38 |
| | pDN620 | 15.41 |
| | | |
| CBH-4D | sf1b | 396.43 |
| | pDN570 | 158.76 |
| | pDN580 | 53.23 |
| | pDN590 | 92.13 |
| | pDN600 | 77.54 |
| | pDN610 | 13.81 |
| | pDN620 | 13.93 |

The upper part of the table (rows entitled "No Ab" and "PE only" represent negative controls to establish the background level of fluorescence in the absence of any E2 protein and in the absence of either primary and secondary antibody (No Ab) or in the presence of only the secondary antibody (PE only). As shown, the background signal is approximately 14. The rest of the table presents fluorescence data obtained for each of the claimed antibodies when tested for their binding to a polypeptide containing either full length E2 (Sf1b) or to selected E2 deletion polypeptides. As is evident from the table, the level of binding to full length E2 and to a number of the E2 deletion polypeptides was significantly above background for each of the

antibodies. However, for each antibody, as the size of the deletion from the N-terminal region increased, a sudden drop in fluorescence signal down to approximately background levels occurred between two of the listed constructs as an additional 10 amino acids was deleted. For example, in the case of CBH-7, binding to pDN490 resulted in a fluorescence of 931.51, while binding to pDN500 resulted in fluorescence of only 24.43.

13. For each of the antibodies, the largest N-terminal E2 deletion that clearly does not contain the epitope to which the antibody binds is highlighted in yellow. For example, CBH-7 shows only background binding to pDN500 (amino acids 500-661). CBH-2, -5, -8C, and -11 show only background binding to pDN430 (amino acids 430-661). CBH-4G shows only background binding to pDN510 (amino acids 510-661). *Thus each of these antibodies fails to significantly bind to a portion of HCV E2 that encompasses amino acids 607-627.*

14. CBH-4B and CBH-4D show background binding to pDN610 (amino acids 610-661). However, they show some binding to pDN600 (amino acids 600-661). Therefore, the epitope to which CBH-4B and CBH-4D bind must be located within positions 384-609, resulting in an overlap of at most 3 amino acids (amino acids 607-609) between the portion of HCV E2 that contains the epitope and amino acids 607-627. *CBH-4B and CBH-4D show significantly greater binding to sf1B (full length E2 protein) than they do to pDN600, indicating that amino acids that lie outside positions 600-661, and thus outside positions 607-627, contribute to the epitopes to which CBH-4B and CBH-4D bind.*

15. As one skilled in the art, I conclude that the E2 deletions for which only background signal was obtained for a particular antibody clearly do not encompass the epitope to which that antibody binds. Because antibodies CBH-7, -2, -5, -8C, and -11 each show only background binding to E2 deletions that encompass amino acids 607-627, these antibodies could not be produced using an epitope defined by position 607-627 as an immunogen. Therefore, CBH-7, -2, -5, -8C, and -11 are distinct from those that would be obtained by using an epitope defined by position 607-627 as an immunogen.

16. As one skilled in the art I further conclude that since CBH-4B and CBH-4D show significantly greater binding to sf1B (full length E2 protein) than they do to a portion of E2 containing amino acids 600-661, the epitope to which these antibodies bind must contain some amino acids that lie outside positions 600-661 and thus outside positions 607-627. Therefore antibodies CBH-4B and CBH-4D could not be produced using an epitope defined by position 607-627 and are therefore distinct from antibodies that would be obtained by using an epitope defined by positions 607-627 as an immunogen.

17. I declare that all statements made herein of my own knowledge are true, and that those statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like are made punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the '720 application or any patents that may issue thereon.

Respectfully Submitted,

Steve K H Foung

Dr. Steven K. H. Foung
Date: Nov. 7, 2005